Applications of Systems Biology Approaches to Understanding Artificial Microbial Consortia and Environmental Communities in the VIMSS Applied Environmental Microbiology Core

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ESPP2 Environmental Stress Pathway Project

DOE GENOMICS:GTL ACCELERATING DISCOVERY FOR ENERGY AND ENVIRONMENT

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INTRODUCTION AND ABSTRACT

Cultivation of single species has been at the central core of experimental microbiology for more than a century but offers only a glimpse into the biology of microorganisms in nature. Communities, not individual species, control the process rates that drive key biogeochemical cycles, including the transformation of environmental pollutants of concern to DOE. Thus detailed studies of model consortia and communities that mediate such processes that will allow for experimental manipulation and in-depth analysis of the fundamental biology underlying such systems are essential for advancing DOE objectives. The GTL Environmental Stress Pathway Project (ESPP2) team, we are pursuing two projects to advance these objectives.

Methods development for environmental mRNA analysis. Current technologies applied to environmental samples for RNA transcriptional profiling include RT-PCR and functional gene microarrays. While tremendous progress has been made in understanding microbial communities due to emergence of these technologies, they bear significant limitations that prevent their application in a high throughput manner to de novo communities. We are developing methods for directly sequencing cDNA from environmental samples utilizing new high throughput (HT) sequence analysis technologies. Since 80% or more of total RNA from bacteria is represented by the rRNA pool, it is crucial to first remove those components as thoroughly as possible without adversely impacting mRNA quality, quantity and composition, prior to HT sequence based screening. We have compared three different methods to remove rRNAs and enrich mRNAs of *D. vulgaris* Hildenborough (DvH) samples. The first method utilizes biotin-modified oligos complementary to conserved regions in 16S & 23S rRNA and specific removal by binding to streptavidin-coated magnetic beads. The second uses a commercially available exonuclease that specifically hydrolyzes rRNAs bearing a 5'monophosphate group. The third method uses two rounds of reverse transcription, where rRNAs are first reverse transcribed with multiple universal primers for 16S & 23S rRNAs and subsequently the RNA/DNA hybrids and cDNA are removed by sequential digestion with RNaseH and DNaseI. We have evaluated these three methods alone and in combination using microarray-based analysis of transcription levels. All three methods are able to significantly enrich mRNA from rRNA without introducing significant biases. Microarray analysis revealed significant differences in measured mRNA levels in only 0.5% to 5% of genes across the genome as compared to controls. Comparisons of microarray results with HT sequencing using the Solexa platform are currently ongoing. After validation, application of these methods could be performed on environmental systems from the Hanford and/or Oak Ridge contaminated sites as part of the VIMSS/ESPP applied environmental core studies, as a compliment the DNA based metagenomic analyses already underway at these sites..

Developing manipulatable, lab based, high order microbial consortia. A practical understanding of how community structure leads to process rates and stability is central to DOE objectives in bioremediation and process control. Although there are numerous theories relating to stability in macroecology, their relevance to microbial communities is mostly untested. To further these studies as part of ESPP2 we are in the initial stages of assembling model microbial consortia in the laboratory that will allow us to study and manipulate community interactions in a controlled manner and test the stress responses of the assemblages. The model organisms now used by the ESPP team will serve for constructing initial consortia, encompassing sulfate-reducers (DvH), iron and uranium reducers (*G. metallireducens*) and methanogens (*M. maripaludis*) along with a cellulose or cellubiose utilizing *Clostridia* species. The genomes of all these strains have been sequenced, gene expression microarrays are available within the group, and the individual organisms can be genetically manipulated which will allow unprecedented toolsets to be applied to these controlled communities not possible in natural systems. Additional methanogens, metal-reducing bacteria from DOE contaminated sites (e.g. *Geobacter, Anaeromyxobacter,* & *Desulfovibrio* sp.) and heterotrophic clostridia that can provide end products of cellulose fermentation (ethanol, acetate and lactate) to the other community members may be added to basic consortia designs. Three member consortia combinations including *C. acetobutylicum* or *cellulolyticum*, DvH and *G. lovelii*, as well as an additional test consortia with DvH, *G. metallireducens*, and three different *M. maripaludis* have been tested in the past few months. Methods for tracking population dynamics of the consortia members such as qPCR and FISH have also been developed and have shown relatively stable assemblages of these species can often be achieved under certain conditions. Consortia studies incorporating 4 and more community members are ongoing.

METHODS FOR ENVIRONMENTAL mRNA PROFILING

mRNA Enrichment

mRNA enrichment is a critical first step for implementing HT sequencing based environmental gene expression profiling. Using RNA harvested from *Desulfovibrio vulgaris*, we tested three strategies for enrichment and their effects on microarray gene expression profiles. These will be compared to HT sequencing results to understand potential biases and limitations of the developed methodologies

Methods Tested

Subtractive Hybridization: Utilizes biotin modified oligos complementary to conserved regions in 16S & 23S rRNA and subtractive hybridization with streptavidin-coated magnetic beads.

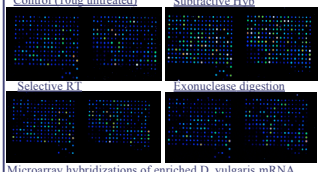
Exonuclease Digestion: Uses a newly available exonuclease that specifically digests rRNAs that bear a 5'monophosphate group.

Selective Reverse Transcription: Uses two rounds of reverse transcription. First rRNA is reverse transcribed with multiple universal primers; RNA/DNA hybrids and cDNA removed by digestion with RNaseH & DNaseI; then enriched mRNAs reverse transcribed using random primers.

Conclusions

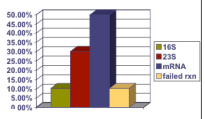
- All three methods were able to significantly enrich mRNAs without introducing systematic biases into microarray based expression profiles and some showed improved sensitivity
- Exonuclease digestion alone was very sensitive to RNA quality and not suitable for the desired environmental application
- No single method was able to eliminate rRNAs entirely
- Combined protocols allow for >50% mRNA enrichment
- The Illumina-Solexa sequencing is currently being applied to experimental samples for comparison to microarray based analyses

Control (10ug untreated) | Subtractive Hyb | Selective RT | Exonuclease digestion



Microarray hybridizations of enriched D. vulgaris mRNA derived from each method. Each of methods give comparable results to 10ug of unenriched starting material using our standard labeling and hybridization procedures.

cDNA enrichment in small library tests



Above. No single method was able to completely remove rRNAs from cDNA libraries. Using a combined method we were routinely able to reduce rRNAs to <50%.

cDNA Amplification

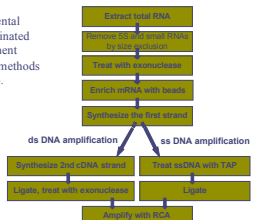
mRNAs are often in low abundance in environmental microbial communities, especially highly contaminated systems. Therefore we aim to couple the enrichment methods described on the left with amplification methods based on phi29 polymerase (RCA amplifications).

Methods Tested

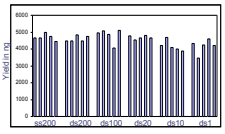
This part of the project has focused on combining and optimizing a string methods for enrichment of mRNA with two variants of a phi29 polymerase amplification using either circularized single stranded or double stranded cDNAs as a template.

Conclusions

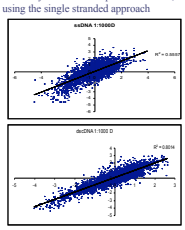
- Using single stranded or double stranded templates phi29 can efficiently amplify cDNAs in the 200 to 1KB range over 10000 fold in 4 hr reactions
- Double stranded amplifications tend to result in more efficient and more unbiased amplifications than single stranded when compared to unamplified controls via microarray hybridizations.



Above. Diagram of the experimental procedure for cDNA amplification. **Below.** Normalized log expression ratios comparing single stranded (top) and double stranded amplifications (bottom) to unamplified enriched cDNAs from log phase DvH cells. Many fewer transcripts are detected, with higher variability using the single stranded approach

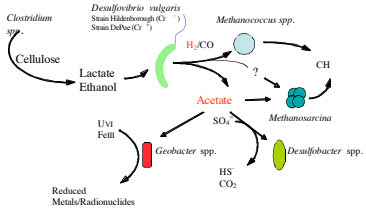


Left. Amplification yields of 5 replicate single stranded (SS) and double stranded (DS) reactions beginning from various amounts of starting total RNA material (in ng)

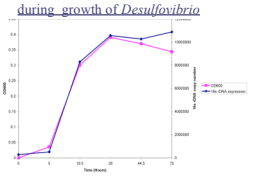


ESTABLISHMENT OF HIGHER ORDER MODEL CONSORTIA

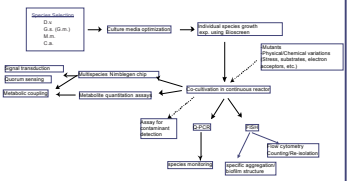
Potential consortia design and hypothesized relationships



Test of QPCR tracking of rDNA copies during growth of *Desulfovibrio*



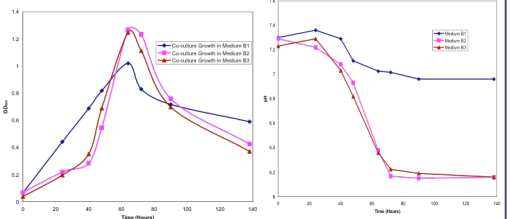
Proposed Multi-Species Work Flow: Techniques, Experiments and Products



In the new phase of the VIMSS/ESPP2 project begun this year, we are working to develop model laboratory consortia that will allow us to use the tools of systems biology developed for environmental microorganisms in this project and others, an apply them to understand the interrelationships between co-occurring species.

Progress

- Initial efforts have focused heavily on both species and strain selection, and basal media formulations that will support the growth of the basic consortia members to which additional complexity and competitors can be added.
- Primary experimental tri-cultures of *Clostridia*, *Desulfovibrio* and *Geobacter* sp. as well as *Desulfovibrio, Geobacter* and *Methanococcus* sp. have been established in both batch and chemostat systems.
- QPCR methods have been developed and FISH probes are being tested for tracking basic population dynamics of the cultures.



Above. Growth of batch co-cultures containing *C. cellulolyticum, G. sulfurreducens, D.vulgaris* in 3 variants of B3A basal Medium; Medium B1 contains cellobiose, fumarate, acetate and lactate; Medium B2 contains cellobiose, fumarate and lactate; Medium B3 contains cellobiose, fumerate and a low concentration of lactate. **The left figure** represents the growth curve of the co-culture in all the 3 mediums. **The right figure** indicates the change in the pH for the corresponding time points.

CONCLUSIONS AND FUTURE WORK

When fully developed and deployed, together these studies will enable us to do indepth analysis of stress mechanisms within environmental and model consortia systems, and understand how the detailed mechanisms outlined using pure culture laboratory systems within the Functional Genomics Core of the VIMSS/ESPP project, translate into the relationships and activities observed in more complex constructed consortia as well as ultimately into environmental microbial communities.

ACKNOWLEDGEMENT

ESPP2 (MDCASE) is part of the Virtual Institute for Microbial Stress and Survival (VIMSS) supported by the U. S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics Program:GTL through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy.